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'Rivulus marmoratus': A Unique Fish Useful in Chronic Marine Bioassays of Halogenated Organics

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16. ABSTRACT

Results are reported for chronic marine bioassays exposing the cyprinodontid fish Rivulus marmoratus, to 2,3,4,6-tetrachlorophenol. Purpose of the study was to illustrate the value of R. marmoratus as a chronic bioassay animal. Results demonstrated the relative insensitivity of R. marmoratus to chronic toxic effects of the chemical, but pathological changes were observed. Fish at least two-months-old showed gill damage ranging from severe (at the highest exposure concentration) to moderate and slight (at the lowest concentration). Such pathological changes might not be seen in chronic laboratory experiments with organisms of greater sensitivities.

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CHAPTER 72

Rivulus marmoratus: A UNIQUE FISH USEFUL IN CHRONIC MARINE BIOASSAYS OF HALOGENATED ORGANICS

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INTRODUCTION

The cyprinodontid fish *Rivulus marmoratus* is a synchronous hermaphrodite with internal self-fertilization.[1] This natural mode of reproduction has resulted in isogenic lines (clones) of homozygous individuals.[2,3] *R. marmoratus* therefore provides a unique opportunity to eliminate genetic variability as a source of experimental error. In addition, it is extremely hardy under laboratory conditions and large numbers may be cultured in a relatively small space.

Our investigations to date have involved long-term, full life-cycle exposure of *R. marmoratus* to the following chemicals: pentachlorophenol, 2, 3, 4, 6-tetrachlorophenol, 2, 3, 5-trichlorophenol, bromoform and dibutyl phthalate. As positive controls for tumorigenic response, we have also performed chronic exposures with diethylnitrosamine and dimethylnitrosamine. Since our data on most of these compounds are preliminary, we will present only our findings to date with 2, 3, 4, 6-tetrachlorophenol and thereby hope to illustrate the value of *R marmoratus* as a chronic bioassay animal.

Tetrachlorophenols are used as fungicides but also may comprise up to 12% of technical-grade pentachlorophenol which, in turn, is the second most

heavily used biocide in the United States, with an annual production in excess of 52 million lb.[4] In the marine environment, these compounds are used in oil and gas exploration as biocides in drilling muds and well-completion fluids.[5]

MATERIALS AND METHODS

Culture Methods

Adult *R. marmoratus* were held singly in 4.5-in. (11.4-cm)-diam stackable culture dishes filled with 200 ml of synthetic seawater (14 °/oo). A Teflon® screen suspended off the bottom of each dish by a glass ring (10 cm diam) prevented the adults from eating the majority of their eggs. Dishes were checked for eggs three times weekly, and viable eggs were incubated singly in 2-in. (5-cm)-diam stackable culture dishes. All studies and all phases of the life cycle were maintained at a temperature of 25 ± 0.5°C, a salinity of 14 ± 1 °/oo and a photoperiod of 14 hr. Adults were fed frozen brine shrimp *(Artemia)* three times a week and postlarvae and juveniles were fed newly hatched *Artemia* nauplii.

Experimental

Sublethal water concentrations were chosen as fractions (0.20, 0.10, 0.05) of static 96-hr LC_{50} tests on newly hatched larvae of *R. marmoratus*.[6] Fish larvae are generally more sensitive to toxic effects than are either adults or eggs. Probit analysis was used to evaluate dose-mortality curves.

For chronic studies, six *R. marmoratus* were randomly selected for each of the three sublethal concentrations, the clean water control and the acetone control. All fish were held singly, and all solutions (contaminated and control) were made fresh and changed weekly.

Technical grade 2, 3, 4, 6-tetrachlorophenol was purchased from Aldrich Chemical Company. Final water concentrations were made from an acetone stock solution. Final concentrations of acetone did not exceed 0.70 mg/l.

The parental adults were exposed from October 20, 1978 until June 20, 1979, whereas the offspring were exposed from conception through August 20, 1979. Gross examinations for fin erosion were made on all surviving offspring, whereas gross examinations for gill damage were made from each dose and the controls on ten randomly selected offspring older than 2 months. Histological analyses were performed where appropriate. During the course of the study, records were kept on adult, larval and embryo mortalities and gross abnormalities. All embryos were staged and any developmental anomalies were described and recorded.[7]

RESULTS AND DISCUSSION

Experimental

The 96-hr LC_{50} of 2, 3, 4, 6-tetrachlorophenol on larvae of *R. marmoratus* was 1.10 mg/l. The chronic exposures were therefore set at 0.22, 0.11 and 0.055 mg/l, which corresponds to 20, 10 and 5% of the LC_{50} respectively.

The overall results of the chronic exposure of *R. marmoratus* through a full life cycle are summarized in Table I. The mortality of parental adults was the same for the controls as for exposed fish. Percent hatch was also comparable among controls and all exposures, indicating no significant effect of tetrachlorophenol on embryo mortality. There was no significant pattern of embryo abnormalities. There was, however, a clear dose response in percent survival of the F_1 generation. Gross and histological analysis of the survivors indicated severe fin and gill erosion, which also showed a dose response.

Damage to the gills was apparent on examination under a dissecting microscope. All fish examined were at least two months old and all showed gill damage ranging from severe (at the highest level) to moderate and slight

Table I. Survival and Gross Pathologic Changes in *Rivulus marmoratus* During Chronic Exposure to 2,3,4,6-Tetrachlorophenol from October 20, 1978 to August 20, 1979

	Nominal Water Concentration (mg/l)				Acetone
	0.220	0.110	0.055	0.00	0.7
Number of Adults 20 Oct. 78	6	6	6	12	6
Number of Adults 20 June 79[a]	5	5	5	10	6
Hatch (%)	49.4	53.9	51.7	47.3	48.9
Number of Offspring	52	101	62	141	44
Number of Survivors	19	43	42	99	26
Survival of Offspring (%)	36.5	42.6	67.7	70.2	59.1
	Survivors Affected (%)				
Fin Erosion	42.1	25.6	14.3	0.0	0.0
Gill Erosion	100.0[b]	100.0[c]	100.0[c]	0.0	0.0

[a] Parental exposure terminated June 20, 1979.
[b] Extensive necrosis of lamellae and filaments.
[c] Lamellar necrosis with slight to moderate filament damage.

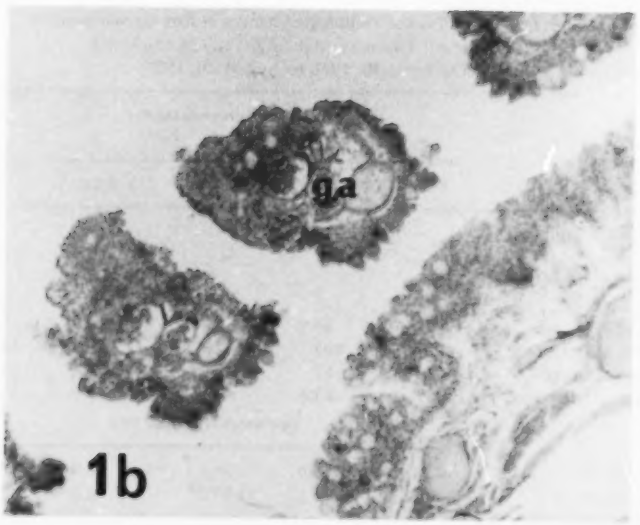

Figure 1. (a) Photomicrograph of a cross section through the gill arches (ga) of (a) *Rivulus marmoratus* showing normal gill filaments (gf) and gill lamellae (gl). 100X and (b) *R. marmoratus* exposed to 2,3,4,6-tetrachlorophenol (0.220 mg/l) for entire life (4 months). Note complete absence of gill filaments and lamellae. 100X.

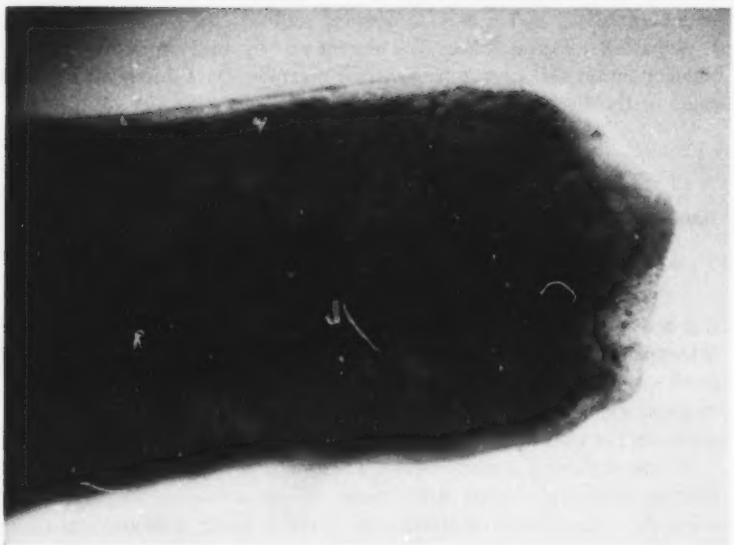

Figure 2. Photomicrograph of the caudal region of *R. marmoratus* exposed to 2,3,4,6-tetrachlorophenol (0.220 mg/l) for entire life (5 months). Note complete absence of fin structures. 12X.

(at the lowest level). All controls had normal gills. In the most severe cases there was complete loss of gill lamellae and nearly complete loss of filaments (compare Figure 1a with 1b). In less-severe cases the terminal portions of the filaments were stunted and the lamellae were irregular in shape.

Fin erosion was observed to continue in exposed fish until the complete fin structure was lost (as in Figure 2). Pectoral fins usually showed signs of erosion first, followed by median fins. The dose response clearly shows that tetrachlorophenol was the primary causative agent; however, it is not yet known whether secondary causative agents (bacterial or viral) were involved. We are presently investigating this aspect.

Fin erosion has recently been reported in several species of marine fish at three coastal locations: in the New York bight,[8] off southern California,[9] and in the estuary of the Duwamish River, Seattle, Washington.[10] In each case the highest incidence of fin erosion was observed in areas of heavy contamination by domestic and industrial pollutants. Although the investi-

gators were not able to determine the primary causative agent, a high correlation between the occurrence of the disease and the presence of various pollutants (heavy metals and halogenated organics) led them to suggest these as probable etiological factor. The present study strongly corroborates the contention that certain halogenated organics play a dominant role in the cause of the disease.

Bioassay System

There are numerous advantages to the *R. marmoratus* bioassay system that are available from no other systems, marine or freshwater. The system is inexpensive, easy to maintain and large stocks may be kept in a relatively small space. In addition, all culture materials are made of either Teflon or glass, which is essential to avoid contamination by such ubiquitous substances as phthalate ester plasticizers.

R. marmoratus is a small fish ($\leq$ 1.5 g) which, in nature, inhabits mangrove swamps where water levels rarely exceed 15 cm and where periodic drying occurs.[11] Therefore, it is preadapted to life in static, shallow-water conditions where there may be extreme fluctuations in salinity. Again, its mode of reproduction is internal self-fertilization, and sexual maturity is attained in about 15 weeks. Sexually mature fish will produce a constant supply of embryos throughout the year under appropriate conditions of day length and temperature, although highest production is during late spring and summer.

Possibly the greatest advantage of *R. marmoratus* is its within-clone genetic uniformity. As a consequence of natural self-fertilization, all individuals are homozygous and three isogenic lines (clones) have been identified.[1-3] Clearly, with the elimination of genetic variability as a source of experimental error, an experimenter may use smaller sample sizes and attain greater uniformity of results.

The results of this study also illustrate the relative insensitivity of *R. marmoratus* to chronic toxic effects. Few fish species can tolerate partial or complete loss of gill lamellae because these are the site of respiratory gas exchange. *R. marmoratus*, however, is capable of breathing air, apparently through the skin, and may be kept out of water in a humid atmosphere for as long as 14 days.[11] The advantages of an experimental animal relatively insensitive to toxic effects are clear. Pathologic changes such as tumorigenic response and those responses described here might never be seen in chronic laboratory experiments with organisms of greater sensitivities.

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